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REVIEW ARTICLE

Emerging roles of hypoxia-inducible factors and reactive oxygen species in cancer and pluripotent stem cells

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Abstract Eukaryotic organisms require oxygen homeostasis to maintain proper cellular function for survival. During conditions of low oxygen tension (hypoxia), cells activate the transcription of genes that induce an adaptive response, which supplies oxygen to tissues. Hypoxia and hypoxia-inducible factors (HIFs) may contribute to the maintenance of putative cancer stem cells, which can continue self-renewal indefinitely and express stemness genes in hypoxic stress environments (stem cell niches). Reactive oxygen species (ROS) have long been recognized as toxic by-products of aerobic metabolism that are harmful to living cells, leading to DNA damage, senescence, or cell death. HIFs may promote a cancer stem cell state, whereas the loss of HIFs induces the production of cellular ROS and activation of proteins p53 and $p16^{Ink4a}$, which lead to tumor cell death and senescence. ROS seem to inhibit HIF regulation in cancer cells. By contrast, controversial data have suggested that hypoxia increases the generation of ROS, which prevents hydroxylation of HIF proteins by inducing their transcription as negative feedback. Moreover, hypoxic conditions enhance the generation of induced pluripotent stem cells (iPSCs). During reprogramming of somatic cells into a PSC state, cells attain a metabolic state typically observed in embryonic stem cells (ESCs). ESCs and iPSCs share similar bioenergetic metabolisms, including decreased mitochondrial number and activity, and induced anaerobic glycolysis. This review discusses the current knowledge regarding the

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emerging roles of ROS homeostasis in cellular reprogramming and the implications of hypoxic regulation in cancer development.

Introduction

Oxygen homeostasis is essential for multicellular organisms to maintain proper function in cellular metabolism and bioenergetics. Oxygen deprivation creates significant stress that induces cellular responses, which lead to the generation of new vasculature to increase oxygen supply and glycolytic capability [1]. An appropriate stress response is required for cells to maintain homeostasis. Low oxygen tension (hypoxia) maintains the undifferentiated state of embryonic, hematopoietic, mesenchymal, and neural progenitor cells [2]. Because complete absence of O_2 (anoxia) results in cell death [3], cells must respond quickly to decreasing O_2 levels before reaching an anoxic state. During hypoxia, cells activate the transcription of genes that induce the adaptive response to supply O_2 to tissues by angiogenesis and erythropoiesis [4]. Reactive oxygen species (ROS), such as the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (HO^-), consist of radical and nonradical oxygen species formed by the partial reduction of oxygen. ROS have been understood to be toxic by-products of aerobic metabolism, leading to cell damage [5].

It seems that the most important transcription factors in the hypoxic response are the hypoxia-inducible factors (HIFs) [6], which mediate transcriptional response to localized hypoxia, in both normal tissues and cancer cells. HIFs consist of an oxygen-labile α-subunit (HIF-α) and a stable β-subunit (HIF-β), and aryl hydrocarbon receptor nuclear translator. There are three isoforms of HIF-α, namely, HIF-1α, HIF-2α, and HIF-3α [4,6]. Hypoxia regulates the undifferentiated state in various stem cell populations [2]. HIFs directly regulate the expression of transcription factors implicated in stem cell self-renewal and multipotency, and induce human embryonic stem cell (hESC) signatures in cancer cells [7]. Hypoxia also enhances reprogramming of fibroblasts into induced pluripotent stem cells (iPSCs) [7].

This review highlights the current understanding of emerging insights into the intricate roles and functions of HIFs and ROS in tumor growth, apoptosis, and senescence, and their roles in reprogramming cells into PSCs by repression of tumor suppressor genes. Moreover, we will discuss several crucial roles of the HIF signaling in the regulation of stem cell self-renewal and its pluripotency.

Regulation of cancer development by HIFs and ROS

It has been demonstrated that HIF-2α promotes hypoxic cell proliferation by enhancing c-Myc transcriptional activity [8]. During oncogenesis, HIFs activate genes that induce tumor invasion and migration [9], and the cancers can grow from cancer stem cells, which are self-renewing tumor cells, propagating tumors phenotypically similar to the parental tumor [10,11]. Hypoxia and HIFs may contribute to the maintenance of putative cancer stem cells [12].

The glycolysis and the consumption of glucose are promoted primarily by HIF-1α, whereas fatty acid storage is promoted by HIF-2α. Both factors inhibit mitochondrial consumption and oxidation of carbon, leading to a decreased production of adenosine triphosphate (ATP) through oxidative phosphorylation and less ROS as a by-product [4,13,14]. It has been known that mitochondria plays a role in controlling ATP production through the electron transport chain, calcium homeostasis, apoptosis, and cell signaling [15]. Instead, ROS have been known to increase HIF-α stability in inflammatory cells [16]. Moreover, accumulation of HIFs is the result of increased generation of ROS by nicotinamide adenine dinucleotide phosphate (reduced) (NADPH) oxidase [17]. Therefore, it should be important to reveal the underlying mechanisms between regulation of HIFs and ROS production in cellular metabolism, oncogenesis, and stem cell biology. ROS, such as O_2^-, H_2O_2, and HO^-, are implicated in the pathophysiology of various diseases, including cancers [18]. The hypoxic condition leads to increased ROS production using an ROS-sensitive fluorescence resonance energy transfer probe containing a redox-sensitive linker. Guzy et al [19] found compelling evidence for increased H_2O_2 production from mitochondria during hypoxia.

The oncogene *Ras* is the most highly mutated oncogene found in human cancer cells [20]. Overexpression of *Ras* has been linked to vascularization of tumors, and activated *Ras* has also been associated with the production of ROS [21]. Thus, oxidative stress is assumed to play a key role in tumor angiogenesis and cancer progression [22]. For example, ROS generated by an NADPH oxidase has already been shown to induce molecular markers of angiogenesis, such as vascular endothelial growth factor-A (VEGF-A) [23]. Increased ROS production by *Ras*-induced Nox1 (a member of the NADPH oxidase family) is also required for oncogenic transformation [24]. Interestingly, the activator protein-1 (AP-1) transcription factor Jun D reduces the activity of an oxygen sensor in the organism by regulating the expression of genes that function in response to oxidative stress and H_2O_2 metabolism [18]. By limiting ROS generation and HIF-1α protein stability, Jun D decreases the transcription of VEGF-A, displays anti-angiogenic properties, and can counteract *Ras*-mediated oncogenic effects. Similar results were obtained in a previous study in which c-Jun dimerization protein 2 (JDP2), a member of the AP-1 transcription factor family, suppressed cell proliferation during cancer progression and participated

in the maintenance of ROS homeostasis and antioxidation to prevent damage to cells by oxidative stress [25].

The level of ROS is tightly controlled by an inducible antioxidant program that responds to cellular stressors and is regulated predominantly by Nrf2 and its repressor protein, the kelch-like Ech-associated protein 1 [26,27]. In contrast to the acute response of Nrf2, in the steady state, some somatic mutations cause destabilization of Nrf2 and decrease the constitutive transcription of its target genes, indicating that enhanced ROS detoxification and additional Nrf2 functions may be critical for the induction of the antioxidant response. A high glycolytic flux supports the proliferative potential of murine ESCs [28]. Because JDP2 is a member of the stress-induced AP-1 protein family [29], we examined the role of JDP2 in cell proliferation, ROS production, and antioxidant response and then identified the JDP2 transcription factor as a cofactor that enhances the antioxidant-responsive element (ARE) activity [30]. JDP2 bounds to ARE and regulates the ARE-mediated transcription associated with the Nrf2/MafK factors. The Nrf2 is known as a central regulator of the induction of many antioxidant-responsive genes and genes encoding Phase II detoxification enzymes. However, Nrf2 is not a DNA-binding protein and the addition of Nrf2 and MafK leads to the repression of ARE reporter genes. Therefore, the real target molecule to enhance the ARE activity in response to the oxidative stress remains to be identified. Thus, we implicate that JDP2 is one of such molecules to enhance the transcription activity of ARE reporter genes and to inhibit ROS production to form the positive complex with Nrf2/MafK via leucine zipper domains. Therefore, JDP2 acts not only as an AP-1 repressor protein, to suppress cell proliferation and induce cellular senescence during cancer progression, but also participates in the maintenance of ROS homeostasis to prevent cell damage by ROS to maintain the stemness feature [30]. This complex feature of JDP2 is also controlled by hypoxia and HIFs.

Tumor hypoxia is typically associated with poor patient prognosis, partly because low oxygen levels reduce the effectiveness of radiation therapy, which kills tumors by generating ROS [6]. It remains to be clarified whether mitochondrial ROS would activate HIF-1α, which as a feedback mechanism would decrease excessive ROS generation through the expression of cytochrome c oxidase subunit 4 isoform 2 and glycolytic enzyme activity of pyruvate dehydrogenase kinase 1 (PDK1) [31]. HIF activation might prevent excessive ROS production in hypoxic cells by regulating mitochondrial respiration through increased expression of PDK1 and switching of cytochrome c oxidase subunit 4 isoform 14 [32,33]. Under normal oxygen conditions (normoxia; defined as 21% O_2), the HIF-α subunit is hydroxylated at conserved proline residues in the oxygen-dependent degradation domain by prolyl hydroxylases (PHDs) [31].

Recent data also demonstrated that the self-renewal state of human iPSCs may be supported by glycolysis metabolism [34] and by mitochondrial properties similar to those of ESCs, including low mitochondria DNA copy number, immature organelle shape with underdeveloped cristae, and low levels of oxidative stress [35]. Underdeveloped mitochondrial networks and low mitochondrial activity are common indicators of stem cell competence, as reported for primordial germ cells, early embryos, ESCs [36], and iPSCs [35]. Glycolysis might be advantageous compared with mitochondrial respiration, because it provides quick energy supplies, thereby avoiding toxic ROS generation [34]. Solid tumor cells shift from aerobic respiration to glycolysis-based metabolisms as a result of the so-called Warburg effect [37]. It has been demonstrated that hypoxic culture conditions and reduced mitochondrial activity are associated with reduced ESC differentiation and increased generation of iPSCs [38]. Thus, a hypoxic environment and glycolytic metabolism seem to be advantageous for maintaining the stem cell phenotype.

Roles of HIFs in stemness maintenance and cancer progression

It has been demonstrated that HIF-2α suppresses p53 tumor suppressor protein and thereby promotes radioresistance and chemoresistance of tumor cells [39]. Furthermore, HIF-2α has been shown to activate Oct4, and HIF-2α-deficient embryos have a severely reduced number of primordial germ cells [40]. Hypoxia probably regulates the proliferation and differentiation of multiple stem cell populations [6]; low oxygen levels are beneficial for hESCs [6], neural stem cells [41], hematopoietic stem cells [42], and tumor cells [43]. For example, Oct4 and c-Myc, which are the factors identified by Takahashi and Yamanaka [44] for generating iPSCs from differentiated cells, are activated by HIF-2α in a renal carcinoma cell line [45]. Therefore, it is important to determine whether HIFs are required for the acquisition of stem cell fates, and in the mechanism underlying the low oxygen effect in reprogramming of cells [7]. Virus-mediated transduction of Yamanaka factors into cells is now a commonly used method for generating iPSCs [46]. Indeed, a virus infection-induced immune response, such as innate immunity, can result in accumulation of ROS [47]. Therefore, it was recently proposed that a virus infection might be detrimental to the survival of iPSCs because of ROS production [48]. It is also worthwhile confirming a role for ROS in reprogramming via HIF-signaling pathways.

Hypoxia can promote an undifferentiated state in certain populations of iPSCs and cancer progenitor cells [6]. Recent studies have suggested that during iPSC formation, a metabolic switch from aerobic respiration and higher mitochondrial activity to decreased mitochondrial activity and the induction of anaerobic glycolysis needs to take place [49]. The dependency of stem cells on glycolysis to produce ATP could be an adaptation to hypoxic conditions *in vivo*, given that low oxygen tensions seem to be a key feature of the stem cell niche [50]. In addition, HIFs have stage-specific roles during reprogramming of human cells into PSCs [7], and HIF-2α is required during the early iPSC reprogramming process for metabolic switching in human fibroblasts. It has been proposed that the cells undergoing the reprogramming process might have similar characteristics to cells undergoing the progression to aggressive tumor cells. It is worthwhile discussing how these results add an important new perspective to our traditional view of hypoxia, cancer, and PSCs.

HIF-specific therapy

Stem cells were reported to engage scavenger antioxidant enzymatic systems to eliminate the ROS that are regulated by hypoxic niches and several transcription factors, including Nrf2 and the forkhead homeobox type O (FoxO), that activate the transcription of antioxidant enzymes [51]. In addition, stem cells can control oxidative stress to maintain antioxidation (redox homeostasis) through mechanisms whereby they upregulate their own antioxidant systems. Ataxia telangiectasia mutated (ATM) protein kinase may control the intracellular levels of ROS [51]. For example, *Atm* knockout mice were found to have progressive bone marrow failure, caused by a defect in hematopoietic stem cell function, which was associated with elevated ROS [52]. Moreover, *Atm* knockout neural stem cells were impaired by intrinsic elevation of ROS levels [53]. Therefore, those experiments imply that the self-renewal capability of stem cells depends on ATM-mediated redox homeostasis [5]. The signaling of hypoxia affects the crucial pathways, such as bone morphogenetic proteins, Akt/mammalian target of rapamycin, and Notch [4]. Medulloblastoma (MDB) precursor cells probably require hypoxic conditions for *in vitro* development, whereas exposure to 20% oxygen induces tumor cell differentiation and cell death through inhibition of Notch signaling. Moreover, MDB tumor cells undergo neuronal differentiation when treated with γ-secretase inhibitor, which prevents Notch activation [54]. These results suggest that hypoxia modulates Notch signaling in promoting the survival and development of MDB stem cell through HIF-1α stabilization. Studies on overexpression and knockdown of HIF-1α and HIF-2α in the von Hippel–Lindau protein-deficient clear cell renal cell carcinoma cell lines indicate that HIF-2α, but not HIF-1α, is necessary for tumor growth [55,56]. One possible explanation for this contradictory effect is that HIF-1α antagonizes c-Myc function, whereas HIF-2α promotes c-Myc activity [8].

The most advanced HIF-pathway-specific cancer drugs in terms of therapeutic application are PHD inhibitors [4].

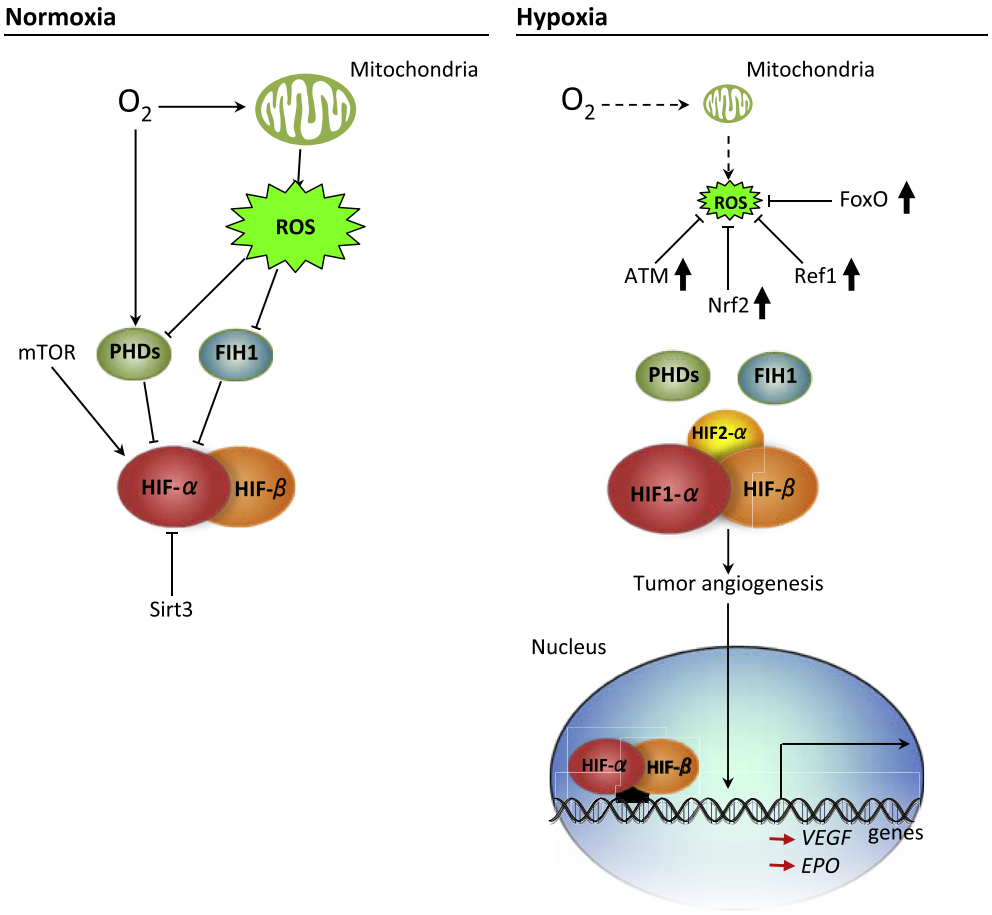

Figure 1. Schematic model showing regulatory mechanism of mitochondrial reactive oxygen species (ROS) and hypoxia-inducible factor (HIF) in tumor angiogenesis. Under normoxic condition, prolyl-hydroxylase domain-containing enzymes (PHD) activity is inhibited by mitochondrial ROS. PHDs and factor inhibiting HIF (FIH) inhibit the expression of HIF-α subunit. HIF-α activity is regulated by sirtuin 3 [85], whereas HIF-α activity is upregulated by mammalian target of rapamycin (mTOR). Under hypoxic conditions, low ROS levels are maintained by redox homeostasis, which is regulated by the antioxidant enzymatic defense systems through the activity of FoxOs, Ref1, Nrf2, and ataxia telangiectasia mutated (ATM). HIF-α stabilization results in the expression of HIF target genes, such as vascular endothelial growth factor (VEGF) and erythropoietin (EPO), with effects on metabolism, tumor angiogenesis, redox homeostasis.

Xenograft tumors grown in *phd2* heterozygotes are less hypoxic and have more functional vessels than those in control mice [57]. In addition to PHD inhibition, HIF-1α adenoviral therapy has shown benefit in models of ischemic disease [58] and limb ischemia in aged and diabetic mice [59]. These findings indicate that the effects of activating the HIF response are a part of disease recovery [4]. Future investigation of the combined effects of HIF-targeted therapy with ROS as an anticancer signaling agent in cancer cells is expected (Fig. 1). VEGF promotes the development of increased vasculature, and is therefore an important protein in the coordination of defense against hypoxia [60]. In response to hypoxia, HIF-1α and HIF-2α regulate genes that affect angiogenic changes such as those for VEGF [61]. VEGF has become an attractive target in the development of anticancer drugs [62]. The activity of HIF can be induced in several transformed cells via oxygen-independent oncogenic signaling pathways, including those regulated by insulin-like growth factor 2/insulin-like growth factor receptor, transforming growth factor-α/epidermal growth factor receptor, and phosphoinositide 3-kinase/Akt [63]. Moreover, JDP2 is also a target of gene therapy for cancer because JDP2 is involved in various functions such as senescence, cell cycle arrest, reprogramming, and tumor suppression.

Effects of p53 signaling on the ROS and HIF-mediated cellular reprogramming

Inhibition of the p53 pathway increases the efficiency of iPSC generation [64—68]. Lebedeva et al [69] reported that the p53 null mouse and p53 knockdown human primary fibroblasts exhibit mitochondrial DNA depletion and mitochondrial mass reduction *in vitro*. Reduced mitochondrial DNA levels, which have been detected in undifferentiated

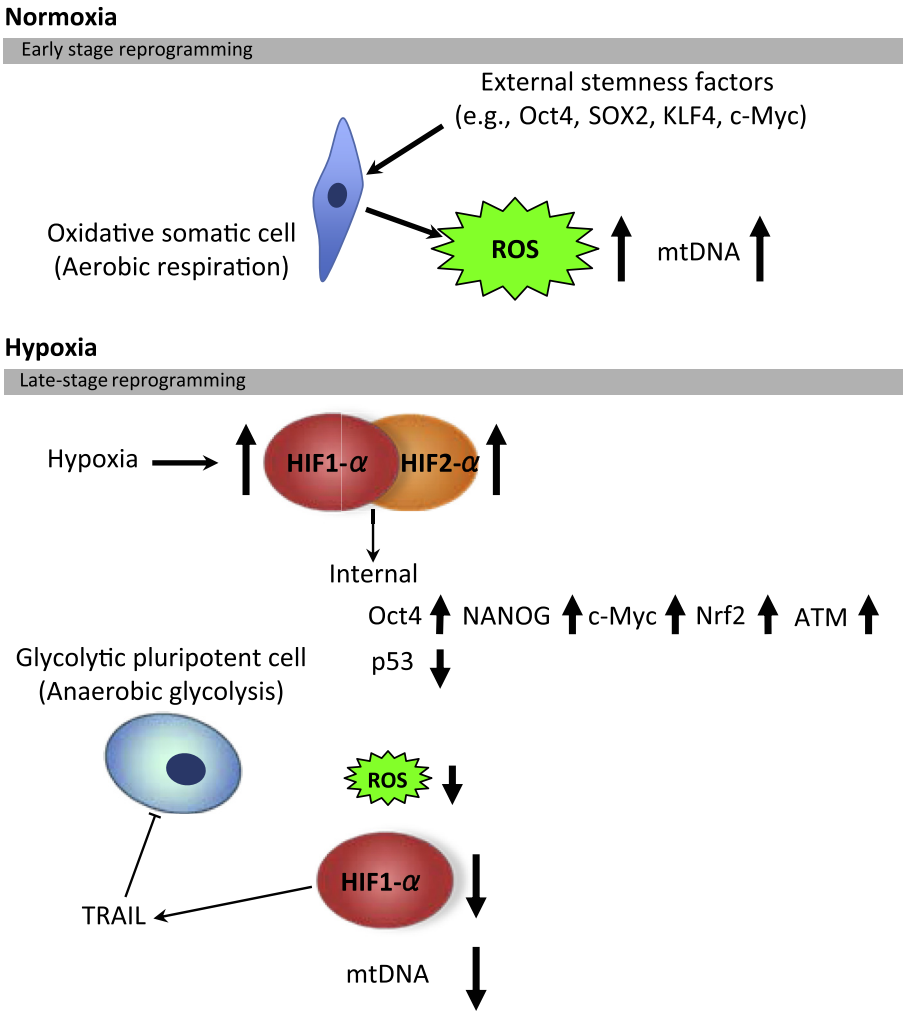

Figure 2. Schematic representation of the roles of reactive oxygen species (ROS) and hypoxia-inducible factor (HIF) in reprogramming to induced pluripotent stem cells (iPSCs) under hypoxic condition. In the early stage of reprogramming, activities of ROS and mitochondrial (mt) DNA are high. Then, through the activation of HIF-1α and HIF-2α under hypoxic conditions, expression of internal stemness genes and antioxidant enzymes increases and that of p53 deceases. Simultaneously, activities of mt DNA, HIF-2α, and ROS signaling decrease. In the late stage of reprogramming to iPSCs, the metabolic switch occurs toward anaerobic glycolysis. The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) represses the process of iPSC reprogramming. ATM = ataxia telangiectasia mutated.

hESCs and iPSCs [55], have been linked to loss of p53 [69]. p53-depleted cells exhibit significant disruption of cellular ROS homeostasis characterized by reduced mitochondrial and cellular superoxide levels and increased H_2O_2 and ROS levels. By contrast, ROS should be suppressed by the antioxidant system (redox homeostasis) of cells during reprogramming [70]. In relation to HIFs, there are opposing effects for the binding of HIF to p53 for cell development. For example, HIF-1α binds to p53, resulting in p53 stabilization and hypoxia-induced cell death [71,72]. By contrast, it has recently been shown that HIF-2α indirectly suppresses p53 activity and promotes radioresistance and chemoresistance in cancer cells [51]. Armstrong and colleagues [35] emphasized the reduction in mitochondrial mass and mitochondrial number during the reprogramming process, which is likely to result in reduced mitochondrial superoxide levels in human iPSCs generated by transduction of Yamanaka 4 factors with NANOG. In addition, they found that human iPSCs clones have antioxidant defense mechanisms similar to those of hESCs, although the precise mechanisms are unclear (Fig. 2).

Conclusion

Hypoxia plays a critical role in maintaining self-renewal and pluripotent capability in cancer and cancer stem cells. Therefore, one can imagine that HIFs should induce dedifferentiation in cancer cells as well as in normal somatic cells. It has recently been shown that hypoxia, but not atmospheric oxygen (21% O_2), can push differentiated hESCs back to the stem cell state [35]. The dedifferentiation process requires histone deacetylase (HDAC) activity through HIFs. HIF-1α can directly interact with HDACs, and hypoxia induces HDAC activity [73].

Reprogramming of differentiated cells to a pluripotent state requires conversion from somatic mitochondrial-dependent oxidative bioenergetics to glycolytic metabolism [7,74]. Mitochondrial regression and deregulation of mitochondrial DNA as a result of reprogramming of cells are consistent with the undeveloped mitochondrial morphology of ESCs [75,76]. iPSCs have diminished basal oxygen consumption and uncoupled oxidative capacity, indicating a shift from oxidative to glycolytic metabolism [7]. ESCs also rely on glycolytic ATP generation, and their pluripotency is maintained under hypoxic conditions [77]. In fact, stimulation by induction of hypoxia or inhibition of the p53 pathway increased reprogramming efficiency [7,78,79]. When oxidative somatic cells are reprogrammed to become glycolytic pluripotent cells, a metabolic change takes place in the early stages of the reprogramming process, and HIF-1α and HIF-2α are essential for this change [7] (Fig. 2).

The stabilization of HIF-1α and HIF-2α in fibroblasts is not sufficient to induce pluripotency because of the repressive effect of HIF-2α in iPSC induction through tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). TRAIL does not induce cell death in hESCs, cancer stem cells, or adult somatic stem cells [7,80]. However, it is detrimental for cancer cells [81,82] and represses the iPSC reprogramming process [7]. Therefore, Mathieu et al [7] suspected that cells undergoing the reprogramming process may have similar characteristics to cells undergoing progression toward aggressive tumor cells [7]. Furthermore, therapeutic application of iPSCs, which are generated using the reprogramming approach, with oncogenic factors might increase the risk of tumor formation. Genetic alterations, including copy-number variations and protein-coding point mutations, were observed during the normoxia reprogramming processes using high-resolution genetic approaches [83,84]. Taken together, these findings suggest that iPSCs have a high tumorigenic potential. In conclusion, the common and shared distinct features of redox homeostasis and hypoxic regulation between iPSCs and cancer stem cells should be clarified to understand the regulation of self-renewal, pluripotency, and cancer development.

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